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J R Yabsley

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

AUSTRALIA

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Johnson & Johnson Research Pty Ltd

PROVISIONAL SPECIFICATION

Invention Title:

siRNA

The invention is described in the following statement:

141476011

siRNA

FIELD OF THE INVENTION

The present invention relates to an siRNA expression vector. More particularly the invention relates to the use of an siRNA expression vector for silencing an expressed gene.

BACKGROUND OF THE INVENTION

The introduction of double stranded RNA (dsRNA) into a range of organisms induces both a potent and specific gene silencing effect. This form of gene suppression by a dsRNA molecule was first observed in *Caenorhabditis elegans* and given the term RNA interference or RNAi (Fire et al 1998). In an attempt to optimise the use of antisense RNA as a tool for controlling specific gene expression in worms, Fire et al (1998) found that dsRNA was more effective than antisense RNA alone. The dsRNA could be generated *in vitro* (Fire et al 1998) or *in vivo* (Tavernarakis et al 2000) and still mediate gene suppression with high specificity. Subsequent studies have shown that dsRNA is an effective inducer of gene silencing in a wide range of eukaryotic organisms and that the mechanism behind this form of gene regulation is most likely conserved throughout evolution (Baulcombe, D. C. (1996) *Plant Mol Biol* 32(1-2), 79-88; Lohmann, J. U., Endl, I., and Bosch, T. C. (1999) *Dev Biol* 214(1), 211-4; Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998) *Proc Natl Acad Sci U S A* 95(25), 14687-92; Cogoni, C., and Macino, G. (1999) *Nature* 399(6732), 166-9; Kennerdell, J. R., and Carthew, R. W. (1998) *Cell* 95(7), 1017-26; Schoppmeier, M., and Damen, W. G. (2001) *Dev Genes Evol* 211(2), 76-82; Baker, M. W., and Macagno, E. R. (2000) *Curr Biol* 10(17), 1071-4; Wargelius, A., Ellingsen, S., and Fjose, A. (1999) *Biochem Biophys Res Commun* 263(1), 156-61).

The molecular mechanism of RNAi has begun to be deciphered using biochemical and genetic approaches in different experimental systems (Hammond, S.M., Caudy, A.A., and Hannon, G.J. (2001) *Nat. Rev. Genet.* 2, 110-19). Presently, RNAi is postulated to involve both an initiation step and an effector step. During the initiation phase, dsRNA is processed by the RNaseIII family nuclease Dicer to produce 21-23 nucleotide duplex siRNAs (small interfering RNAs). These short stretches of dsRNA carry 2 nucleotide 3'-OH overhangs that contribute to the efficacy of gene silencing (Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001) *Genes & Dev* 15:188-200). In the effector phase, these siRNAs are incorporated into a multiprotein complex

called RISC (RNA-induced silencing complex) that targets transcripts by base pairing between one of the siRNA strands and the endogenous mRNA (Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000) *Nature* 404: 293-96). A nuclease activity associated with the RISC complex then cleaves the mRNA-siRNA duplex thus targeting the cognate mRNA for destruction.

In mammalian cells the use of dsRNA to control gene expression has been hampered by the presence of a unique global response mechanism. Mammalian cells exposed to dsRNA longer than 30 base pairs in length trigger a response mechanism involving activation of two key enzymes, dsRNA-activated protein kinase (PKR) and 2'5' oligoadenylate polymerase/RnaseL (Kumar, M., and Carmichael, G. G. (1998) *Microbiol Mol Biol Rev* 62(4), 1415-34). The activation of these enzymes leads to a cessation of protein synthesis and eventually cell death via apoptosis. It was thus anticipated that the introduction of long dsRNA would activate this global response system. However, studies have shown that in both mouse pre-implantation embryos (Svoboda, P., Stein, P., Hayashi, H., and Schultz, R. M. (2000) *Development* 127(19), 4147-4156; Wianny, F., and Zernicka-Goetz, M. (2000) *Nat Cell Biol* 2(2), 70-5) and undifferentiated embryonic stem cells and embryonic carcinoma cells (Yang, S., Tutton, S., Pierce, E., and Yoon, K. (2001) *Mol Cell Biol* 21(22), 7807-16; Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) *Proc. Natl Acad Sci* 98, 14428-14483; Paddison, P., Caudy, A. A., and Hannon, G.J. (2002) *Proc. Natl Acad. Sci.* 99, 1443-1448), the use of *in vitro* generated long dsRNA was able to mediate specific gene silencing. The primary reason for these observations was that these cell systems lack the generalised responses to dsRNA. These results were encouraging but placed particular limitations on the utility of this approach in differentiated mammalian cells.

Following on from observations that the products of the Dicer enzyme could mediate RNAi in Drosophila embryo extracts, it was then demonstrated that chemically synthesised 21 bp siRNAs could be used in a wide range of human and mouse cell lines to induce gene silencing (Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* 411(6836), 494-8; Caplen, N.J., Parrish, S., Imani, F., Fire, A., and Morgan, R.A. (2001) *Proc. Natl. Acad. Sci.* 98, 9742-9747). This approach for transiently controlling the expression of a wide range of different target genes has been demonstrated and is becoming the method of choice for determining gene function in mammalian cells (Hsu, J.Y., Reimann, J. D. R., Sorensen, C.S., Lucas, J., and Jackson, P. K. (2002) *Nature Cell Biol.* 4, 358-366;

Thompson, B., Tonwsley, F., Rosin-Arbesfeld, R., Muisi, H., and Bienz, M. (2002) *Nature Cell Biol.* 4, 367-373).

The ability to express siRNAs that act through the RNAi pathway to regulate specific gene expression allows for a number of functional studies and possibly therapeutic applications. However, new approaches for targeted inhibition of gene expression are required.

SUMMARY OF THE INVENTION

In a first aspect the present invention provides an siRNA expression vector for use in silencing a target gene, the vector comprising a pair of convergent promoters and a DNA molecule positioned therebetween, the DNA molecule comprising a target-specific sequence flanked by two directional transcription terminators, the target-specific sequence comprising a sequence of at least 14 nucleotides having at least 90% identity to a segment of the target gene.

In a second aspect the present invention provides a method for determining a function of a target gene, the method comprising:

preparing an siRNA expression vector comprising a pair of convergent promoters and a DNA molecule positioned therebetween, the DNA molecule comprising a target-specific sequence flanked by two directional transcription terminators, the target-specific sequence comprising a sequence of at least 14 nucleotides having at least 90% identity to a segment of the target gene;

transfecting an effective amount of the siRNA expression vector into a cell to produce a transfected cell; and

detecting one or more phenotypic changes in the transfected cell relative to a control cell.

In a third aspect the present invention provides a method of inhibiting expression of a target gene in an organism, the method comprising:

providing the organism with an siRNA expression vector according to the first aspect.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 Strategy for generating intracellular siRNAs and effect of the expressed siRNAs on transgene expression. (A) The convergent U6 expression cassette encodes sense and antisense RNAs that terminate at directional termination sequences. The complementary RNAs anneal and undergo further Dicer-dependent processing to

produce functional siRNAs. A U6 convergent expression vector containing an EGFP-specific insert (DualU6GFP) reduces dEGFP-mediated cell fluorescence (B and C), dEGFP protein levels (D), and dEGFP RNA levels (E).

Fig. 2 Gene suppression by complementary RNAs expressed from a U6 convergent cassette is Dicer-dependent. (A) The U6 convergent EGFP vector undergoes transcription in EcR293 cells to produce sense and antisense RNAs. (B) Suppression of target gene expression by the DualU6GFP vector requires the co-expression of both sense and antisense RNAs. (C) The DualU6GFP expression vector reduces dEGFP target gene expression in a Dicer-dependent manner. (D) Co-expression of complementary RNAs from the U6 convergent expression cassette does not activate PKR. Hela cells were treated with 0.1 μM calyculin A and serve as a positive control for activated PKR.

Fig. 3 Suppression of p53 protein levels using a convergent U6 expression vector. Plasmids DualU6 and DualU6p53 or p53-specific siRNAs 1 and 2 were transfected into MDA MB 231 cells and at 48 h and 120 h post-transfection p53 and β-actin protein levels determined using western analysis.

Fig 4 Suppression of dEGFP transgene expression using a stably integrated convergent transcription vector. HEK 293 cells were cotransfected with either the pDualU6 vector or pDualU6GFP and the pREP7 plasmid in a 10:1 molar ratio, and cells selected for resistance to hygromycin. Following selection, cells were examined for level of dEGFP-mediated cell fluorescence.

DETAILED DESCRIPTION

The present invention provides a convergent promoter system capable of producing sense and antisense RNAs that mediate gene silencing in mammalian cells through the RNAi pathway. This system can be used to inhibit transgene and endogenous gene expression.

The use of dsRNA as a mediator has distinct advantages over hammerhead and hairpin ribozymes including the presence of a natural cellular protein complex (termed RISC) that binds the expressed dsRNA and mediates interaction with the target mRNA and cleavage of the target mRNA.

In a first aspect the present invention provides an siRNA expression vector for use in silencing a target gene, the vector comprising a pair of convergent promoters and a DNA molecule positioned therebetween, the DNA molecule comprising a target-specific sequence flanked by two directional transcription terminators, the

target-specific sequence comprising a sequence of at least 14 nucleotides having at least 90% identity to a segment of the target gene.

Delivery and transcription of the expression vectors of the present invention in a host cell provides an siRNA specific for a target mRNA having complementarity with the target-specific sequence. The siRNAs of the invention have been shown to be effective modifiers of gene expression.

Preferably the target-specific sequence is at least 19 base pairs in length. More preferably the target-specific sequence is 19 to about 30 base pairs in length. More preferably the target-specific sequence is from 19 to 25 base pairs in length. Most preferably the target-specific sequence is 19 base pairs in length.

The target gene may be any gene of interest, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art.

In a preferred embodiment the target-specific sequence has at least 95% identity, and more preferably is identical, to a segment of the target gene.

The target-specific sequence may be synthetically generated or generated by digesting a viral or pathogenic genome to fragment the starting DNA. Digestion of a viral or pathogenic genome may be achieved by techniques known to those of skill in the art, such as DNAse I digestion or by digestion with restriction enzyme(s). Synthetic sequences may be generated chemically according to known methods such as the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981) Tetrahedron Letts. 22(20):1859-1862, e.g. using an automated synthesiser as described in Needham-VanDevanter et al (1984) Nucleic Acids Res., 12:6159-6168. Purification of the molecule, where necessary, is typically performed by either gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier (1983) J. Chrom. 255:137-149. The sequence can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, Methods in Enzymology 65:499-560.

As used herein, the term "complementary" is used in reference to "polynucleotides" and oligonucleotides" (which are interchangeable terms that refer to a sequence of nucleotides) related by the base pairing rules. For example, the sequence 5'-CTGAG-3' is complementary to the sequence 5'- CTCAG-3'. Complementarity can be partial or total. Partial complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules. Total or complete complementarity is where each and every nucleic acid base is matched with another base according to base pairing rules. The degree of complementarity between

nucleic acid strands has significant effects on the efficiency and strength of hybridisation between nucleic acid strands.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a host organism. Nucleic acid sequences necessary for expression in eukaryotic cells include, for example, polyadenylation signals. In a preferred embodiment the expression vector also incorporates stabilisation elements into the expressed RNA to increase the stability of the RNA. Suitable expression vectors include plasmids, viruses, retrotransposons and cosmids. Preferably the expression vector is suitable for expression in a mammalian cell.

In a preferred embodiment the expression vector is a retroviral expression vector.

In a preferred embodiment the expression vector encodes a selectable marker, for example an antibiotic resistance gene, for selection of cells transfected with the expression vector. More preferably the expression vector encodes the G418 selection marker.

Methods which are well known to those skilled in the art can be used to construct expression vectors of the present invention. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination or genetic recombination. Such techniques are described in Sambrook et al (1989) Molecular Cloning, A laboratory Manual, Cold Spring Harbor Press, Plainview N.Y. and Asubel F M et al (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York N.Y.

Transcription from the convergent promoters of two strands of the resident inserts results in the production of two small complementary RNAs that are capable of hybridising to form an siRNA with two to four base overhangs at their 3' ends.

In a preferred embodiment the convergent promoters are U6 snRNA, H1 or T7 promoters. More preferably the convergent promoters are U6 snRNA promoters.

The expression vector produced according to the methods of the invention are useful in identifying the function of a gene or sequence of interest in an organism.

Accordingly, in a second aspect the present invention provides a method for determining a function of a target gene, the method comprising:

preparing an siRNA expression vector comprising a pair of convergent promoters and a DNA molecule positioned therebetween, the DNA molecule comprising a target-specific sequence flanked by two directional transcription terminators, the target-specific sequence comprising a sequence of at least 14 nucleotides having at least 90% identity to a segment of the target gene;

transfecting an effective amount of the siRNA expression vector into a cell to produce a transfected cell; and

detecting one or more phenotypic changes in the transfected cell relative to a control cell.

Preferably the target-specific sequence is at least 19 base pairs in length. More preferably the target-specific sequence is about 19 to about 30 base pairs in length. More preferably the target-specific sequence is from 19 to 25 base pairs in length. Most preferably the target-specific sequence is 19 base pairs in length.

In a preferred embodiment the convergent promoters are U6 snRNA, H1 or T7 promoters. More preferably the convergent promoters are U6 snRNA promoters.

The present invention provides methods for the modification or identification of one or more functions of a target gene in an organism. The methods of the invention selectively reduce, diminish or destroy the RNA encoded by the target gene in order to render the RNA non-functional while the targeted gene in the host remains intact. These methods therefore employ a "knockdown" strategy to determine or modify gene function instead of the traditional "knockout" methods. The invention is useful for the rapid identification of, for example, disease related genes which may be targeted for the treatment or prevention of disease. The methods of the present invention also have utility in identifying viral or pathogen-derived genes that play a major role in the susceptibility of cells to infection by viruses or pathogens.

The term "transfecting" as used herein refers to the introduction of the expression vector into a cell. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection or biolistics (i.e., particle bombardment). Transfection may be transient or stable transfection. The term "stable transfection" or "stably transfected" refers to the introduction and integration of a transgene into the genome of a transfected cell. The term "transient transfection" or "transiently transfected" refers to the introduction of

one or more transgenes into a transfected cell in the absence of integration of the transgene into the genome of the host cell.

The target gene may be any gene of interest, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art.

Phenotypic changes include the absence or decrease in the level of protein and/or mRNA product from the target gene, altered biochemical properties or altered outward properties of the transfected cell such as morphology. Phenotypic changes can be determined by techniques known to those of ordinary skill in the art including Western blotting, Northern analysis, enzyme-linked immunosorbent assay (ELISA), immunoassay, flow cytometry, microarray or *in situ* hybridization.

It is desirable to express a sufficient amount of siRNA such that substantially all the substrate RNA is cleaved. Such substantial abrogation of substrate RNA expression would facilitate the observation of the effect of depletion of gene function in the organism wherein the siRNA is expressed. While desirable, complete elimination of the substrate RNA is not required by the methods of the invention.

A "control" cell as used herein includes a cell that is untransfected, has been mock transfected, or has been transfected with an "empty vector" such as an expression vector without the DNA molecule insert.

Host cells, such as eukaryotic cells, harbouring the expression vectors described above are also provided by this invention. Host cells may be derived from or contained in any organism. Suitable organisms include, but are not limited to, animal, plant, bacteria, virus, and fungi. Suitable host cells include, but are not limited to, bacterial cells, rat cells, mouse cells and human cells.

The methods of the invention are useful for determining the function of a gene or DNA sequence of interest in an organism by forward genetic approaches including observing the effects of reducing expression of the gene or DNA sequence in the organism or of a homologous gene or DNA sequence in another organism. For example, data presented herein demonstrates that the function of the p53 or EGFP gene in MDA MB 231 breast cancer cells or 293 embryonic kidney cells respectively may be determined by siRNA mediated cleavage of transcripts.

The types of genetic selections that can be used in a forward genetic approach with an RNAi as described herein includes overcoming cell growth arrest by, for example, bypassing p53-mediated growth arrest and apoptosis; identifying new targets involved in chemotherapeutic drug resistance such as overcoming 5-FU-induced growth arrest, apoptosis and senescence; blocking activated signaling

pathways, for example, identifying novel positive and negative regulators of signaling pathways implicated in cancer, such as the TGFβ and Wnt pathways; elucidating resistance to viral and pathogen infection including genetic screens for genes that confer resistance to HIV infection or that interfere with the productive or latent phases of the viral life cycle or genetic screens for genes that interfere with the lifecycle of an intracellular parasite such as plasmodium; synthetic lethality screens to identify gene products whose inactivation leads to cell death, particularly in tumor cells deficient for either the p53 or p16/Rb tumor suppression pathways; identifying genes involved in metastasis, for example using in vivo assays; identifying optimal siRNAs against specific target(s); detecting genes regulating specific promoters; detecting cell cycle regulatory genes, for example using soft agar assays (for anchorage dependent growth) and minimal medium (for growth factor-independent growth), both of which are widely used indicators of cellular transformation in cell culture.

The function of a target gene in a first organism may be determined by reducing expression of a gene in a second organism using the methods of the invention where ethical considerations preclude experimentation on the first organism of interest. Thus, while ethical considerations preclude depleting gene expression in humans in order to determine the function of that gene in a human individual, the ability to reduce expression of a gene sequence that is homologous to the human gene sequence in a model organism (e.g. mouse) permits an initial determination of the function of the gene. The function of the gene may then be further investigated in other model organisms and/or in clinical trials with human subjects.

Furthermore, the ability to express siRNAs that act through the RNAi pathway allows for regulation of expression of genes and therapeutic applications to alleviate disease states resulting from expression of these genes.

Accordingly, in a third aspect the present invention provides a method of inhibiting expression of a target gene in an organism, the method comprising:

providing the organism with an siRNA expression vector according to the first aspect.

The target gene may be a gene derived from a cell of the organism, a transgene, or a gene of a pathogen present in a cell of the organism, or remaining in the cell after infection by the pathogen.

The organism includes, but is not limited to animal, plant, bacteria, virus or fungi.

The expression vector produced according to the method of the first aspect may be provided to the organism by direct introduction, such as direct injection, or introduced by other means known to those of skill in the art including oral introduction or topical application. The expression vector may be introduced into a germ line or somatic cell, stem cell or other multipotent cell derived from the organism and re-introduced into the organism.

The present invention may be used for treatment or prevention of a disease state resulting from expression of the target gene. Disease states include, but are not limited to, autoimmune diseases, inherited diseases, cancer, infection by a pathogen or overexpression of the target gene. Treatment would include prevention or amelioration of any symptom or clinical indication associated with the disease.

Target genes according to the present invention include, but are not limited to, genes involved in chemotherapeutic drug resistance, apoptosis and senescence; genes implicated in cancer including genes involved in metastasis and genes responsible for tumorigenesis.

The present invention also includes pharmaceutical compositions and formulations, which comprise at least one siRNA expression vector of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. The administration can be topical, pulmonary, oral or parenteral.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powders or oily bases, thickeners and the like may be necessary or desirable. Composition and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules satchels or tablets.

The siRNA expression vectors of the present invention can additionally be used to increase the susceptibility of tumour cells to anti-tumour therapies such as chemotherapy and radiation therapy.

Accordingly in certain embodiments of this invention there are provided liposomes and other compositions containing (a) one or more siRNA expression vectors of the invention and (b) one or more chemotherapeutic agents which function by a non-hybridisation mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as taxol, daunorubicin,

dacitinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-flurouracil, floxuridine, methotrexate, colchicine, vincristine, vinlastin, etoposide, cisplatin. See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed., Berkow et al eds., 1987, Rahway, N.J., pp 1206-1228.

The formulation of the therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or diminution of the disease state is achieved. Optimal dosing schedules can be determined from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. In general, dosage is from 0.01 μg to 100 g per kg of body weight and may be given daily, weekly, monthly or yearly.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

All publications mentioned in this specification are herein incorporated by reference. Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

EXAMPLES

Example 1 Constructs and siRNAs

To develop a vector system for expressing siRNAs in mammalian cells compatible with generating RNAi for forward genetic selection, the convergent U6 promoter cassette indicated in Figure 1A was designed. To determine the intracellular

efficacy of this expression cassette for mediating specific gene silencing, the EGFP gene was used as a target.

To construct DualU6 containing convergent U6 promoters, the primers 5'-GCG CAA GCT TAT AGG GAA TTC GAG CTC GGT A-3', and 5'-GCG CTC TAG AGG TGT TTC GTC CTT TCC ACA A-3' were used to PCR amplify the U6+1 promoter region from pTZ(U6+1) (Paul, C.P., Good, P.D., Winer, I, and Engelke, D.R. (2002) *Nature Biotech* 20, 505-508) and the resulting amplicon cloned as a XbaI-HindIII fragment into pTZ(U6+1). The inserts encoding the sense and antisense RNAs were designed to include a 19 bp target-specific sequence (in bold below) flanked by two directional transcription terminators composed of five thymidines. The oligonucleotides used to construct DualU6GFP were 5'- TCG ACA AAA ACG GCA **AGC TGA CCC TGA AGT TTT** T-3' and 5'-CTA GAA AAA **CTT CAG GGT CAG CTT GCC GTT TTT** G-3', while the following were used to construct DualU6p53: 5'-TCG ACA AAA AGA **CTC CAG TGG TAA TCT ACT TTT** T-3' and 5'-CTA GAA AAA **GTA GAT TAC CAC TGG AGT CTT TTT** G-3'. These oligonucleotides were synthesised (Sigma Genosys, Sydney, Australia), annealed and cloned into the SalI and XbaI sites of DualU6.

The RNA oligonucleotides used to form the siRNAs were synthesised by Dharmacon Research Inc (CO, USA) and the sequences were: GFP, 5'-CGG CAA GCU GAC CCU GAA G dTdT (sense); p53(siRNA1), 5'-GAC UCC AGU GGU AAU CUA C dTdT (sense); and p53(siRNA2), 5'-GCA UGA ACC GGA GGC CCA U dTdT (sense). These RNA oligonucleotides were annealed with corresponding antisense strands as described (Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* 411(6836), 494-8).

Example 2 Effect of expressed siRNAs on transgene expression.

Mammalian cells used in this study included the human embryonic kidney cell line EcR293 (Invitrogen, CA, USA) and the human breast cancer cell line MDA MB 231. The construction of the EcR293 cell line expressing the dEGFP gene has been described (Raponi, M., Dawes, I.W., and Arndt, G.M. (2000) *Biotechniques* 28, 840-844). EcR293 cells and their derivatives were maintained in DMEM containing 10% fetal calf serum supplemented with glutamine, streptomycin and penicillin. MDA MB 231 cells were grown in RPMI containing 10% fetal calf serum supplemented with glutamine.

Cells were seeded into 6 well plates 24 h prior to transfection. For all transfections, a total of 4 μg of plasmid DNA or 20 μM of siRNA was delivered using Lipofectamine 2000 (Invitrogen, CA, USA)) according to the manufacturer's instructions. Cells were harvested at 24 h and 48 h for flow cytometry analysis of EGFP expression (Becton Dickinson, USA). Fluorescent microscopy was performed using a fluorescence microscope (Nikon, Japan) with a B-2H filter cube.
A U6 convergent expression vector containing a EGFP-specific insert (DualU6GFP) was constructed and co-transfected with the pEGFP-N1 plasmid and the lacZ expression vector pSVβ into 293 embryonic kidney cells. Cells receiving DualU6GFP displayed a 40% reduction in cell fluorescence compared with cells transfected with the DualU6 control vector.

To further examine the utility of the dual U6 promoter, and the mechanism by which this vector regulated gene expression, the DualU6GFP plasmid was delivered to 293 cells containing a stably integrated destabilised EGFP (dEGFP) transgene. As shown in figure 1B, cells transfected with DualU6GFP displayed a reduction in dEGFP-mediated cell fluorescence with the level of reduction in fluorescence equal to that of the synthetic EGFP siRNA at 48h post-transfection. Consistent with the requirement for expression of the sense and antisense RNAs from DualU6GFP, gene silencing via this vector displayed a 24h delay compared with a synthetic siRNA targeted to the same region of the dEGFP mRNA. The reduction in cell fluorescence exhibited by cells containing the DualU6GFP plasmid was confirmed using fluorescence microscopy (Figure 1C). As with the synthetic siRNAs, the residual population displaying cell fluorescence most likely represents cells that have not been transfected with the expression plasmid.

To examine the utility of the DualU6GFP expression system in long term regulation of gene expression in mammalian cells, either the pDualU6GFP plasmid, or the pDualU6 vector, was co-delivered with pREP7 (containing the marker conferring resistance to hygromycin) to HEK 293 cells expressing the dEGFP transgene. Following selection for cells stably maintaining the DualU6GFP plasmid, cells were examined for dEGFP-mediated cell fluorescence. As shown in figure 4, cells containing the DualU6GFP plasmid displayed a significant reduction in cell fluorescence compared with cells receiving the DualU6 control vector. This result indicates that the convergent expression cassette described can be used to mediate long term regulation of gene expression in mammalian cells.

It has been reported that shRNAs, or co-expression of small antisense and sense RNAs, produce specific gene silencing by processing to siRNAs. To determine the mechanism of action of the DualU6GFP expression system, transfected cells were examined for dEGFP protein levels, dEGFP mRNA levels and the presence or absence of small RNAs encoded by the U6 convergent expression vector containing an EGFP-specific insert.

Western analysis was performed as follows: cell lysates were prepared using RIPA buffer supplemented with protease inhibitors aprotonin (1 µg/ml), leupeptin (10 µg/ml) and DMSF (100 µg/ml). Total protein was loaded onto 4-12% Bis-Tris agarose gels (Invitrogen, CA, USA), separated by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. The antibodies used for detection of specific proteins in the Western analysis included: GFP, mouse polyclonal (Clontech), PKR monoclonal (Cell Signaling), PKR phospho rabbit polyclonal (Cell Signaling), p53 mouse monoclonal (Oncogene Research Products) or β-actin mouse monoclonal (Sigma) antibodies. Secondary antibody detection was performed using either the goat anti-mouse horseradish peroxidase (HRP)-linked or the goat anti-rabbit HRP (SantaCruz), followed by visualisation using the luminol/enhancer chemiluminescent substrate (Amersham Pharmacia Biotech, Piscataway, NJ).

Western analysis showed that the dEGFP protein levels were reduced in cells expressing the siRNA from the U6 convergent expression vector and that this effect was specific (Figure 1D). The level of suppression of the dEGFP protein was equivalent to that mediated by delivery of synthetic siRNAs. An examination of dEGFP target mRNA levels indicated that both the synthetic siRNAs and those expressed from the U6 convergent plasmid reduced target mRNA (Figure 1E). This latter result suggests that DualU6GFP produces siRNAs capable of mediating turnover of the target mRNA, an observation consistent with the mechanism of RNAi.

Example 3 Gene suppression by complementary RNAs expressed from a U6 convergent cassette is Dicer-dependent.

To further confirm that the DualU6GFP plasmid maintains the potential to produce siRNAs, the transcripts expressed from this plasmid were identified using northern blot analysis.

RNA for RNA analysis was isolated using Trizol (Invitrogen, CA, USA) and immobilised onto nylon membrane (Invitrogen, CA, US), for detection using standard probe hybridisation. For the detection of small antisense and sense RNAs encoded by

DualU6GFP, the following oligonucleotides were end-labelled and hybridised to these membranes at 37°C for 1h: 5'- TCG ACA AAA ACG GCA AGC TGA CCC TGA AGT TTT T-3' or 5'-CTA GAA AAA CTT CAG GGT CAG CTT GCC GTT TTT G-3'. Membranes were analysed using a phosphorimager (Molecular Dynamics, USA) and an ImageQuant software package (Molecular Dynamics, USA).

As shown in figure 2A, bands of the expected length were observed only in cells containing the DualU6GFP plasmid and not in vector controls. In addition, using strand-specific probes, it was possible to show that within the cells containing the U6 convergent EGFP vector both the antisense and sense RNAs were present. The sizes of the transcripts confirmed that the directional terminators were operative and that U6-directed transcriptional machinery efficiently truncated the antisense and sense transcripts within the convergent transcription unit. The above results indicate that the use of U6 convergent promoters in a single expression cassette can produce sense and antisense RNAs that mediate specific gene suppression in a manner consistent with RNAi.

To demonstrate the necessity for convergent U6 promoters in the DualU6GFP vector, and therefore the expression of both sense and antisense RNAs, to mediate suppression of the dEGFP target gene, derivatives of this plasmid containing only a single U6 promoter were constructed. These vectors were designated pU6GFPS and pU6GFPAs and were expected to encode small sense and antisense EGFP RNAs under control of the U6 promoter, respectively. Each of these plasmids was used to transiently transfect 293 cells expressing the dEGFP transgene. Cell populations were then analysed for dEGFP-mediated cell fluorescence. This analysis indicated that the expression of either sense or antisense EGFP strands alone was insufficient to suppress the dEGFP gene, and that full inhibition of this target gene required the co-expression of both strands within the same cell (Figure 2B).

Given that the cells co-expressing the sense and antisense EGFP RNAs displayed many of the hallmarks of RNAi, the issue of whether gene silencing occurred through formation of dsRNA was determined. Toward this end, the Dicer siRNA was utilised as a tool to determine if the observed suppression was Dicer-dependent (Hutvagner et al (2001) *Science* 293,834-838). 293 cells expressing the dEGFP transgene were transfected with DualU6GFP in the presence and absence of the synthetic siRNA specific for Dicer. As shown in Figure 2C, the Dicer siRNA completely reversed the reduction in cell fluorescence mediated by the EGFP-specific U6 convergent plasmid. In contrast, cells transfected with both the synthetic EGFP-

and Dicer-specific siRNAs still displayed a reduction of cell fluorescence, as the mechanism of synthetic siRNAs is Dicer-independent. These results suggest that the small sense and antisense RNAs encoded by DualU6GFP anneal to form dsRNA that is processed by Dicer into authentic siRNAs. It is most likely that gene silencing is then directed by these processed siRNAs.

It has been proposed that dsRNA greater than 30 base pairs in size induce a global response that results in activation of the double-stranded RNA-specific protein kinase PKR (Paddison, P., Caudy, A. A., and Hannon, G.J. (2002) *Proc. Natl Acad. Sci.* 99, 1443-1448). To eliminate PKR activation as being responsible for the gene silencing observed using this unique expression system, the levels of both total PKR and activated PKR were examined in 293 cells receiving the DualU6GFP plasmid. This analysis indicated that co-expression of the sense and antisense EGFP RNAs and formation of dsRNAs did not activate PKR (Figure 2D), suggesting that the observed gene silencing effect was specific and not related to this global response mechanism.

Example 4 Suppression of p53 protein levels using a convergent U6 expression vector.

Whether the U6 convergent promoter system could be used to control the expression of endogenous genes in mammalian cells was determined. For this purpose, the TP53 gene that encodes the p53 tumor suppressor protein was chosen as a target. To this end, a U6 convergent expression vector was constructed containing an insert encoding a p53-specific siRNA. The target site selected was identical to that reported earlier for synthetic p53-specific siRNAs (Brummelkamp, T.R., Bernards, R., and Agami, R. (2002) *Science* 296, 550-553). The p53-specific U6 convergent expression plasmid, DualU6p53, was transfected into MDA MB 231 breast cancer and 293 cells and 48h post-transfection cells were harvested and analysed for p53 protein levels. As shown in Figure 3, delivery of the DualU6p53 plasmid resulted in a significant and specific reduction of p53 protein. This result indicates that the U6 convergent promoter system can be used to effectively suppress the expression of endogenous genes through RNAi in mammalian cells.

The system described provides a novel alternative expression modality to shRNA-expressing plasmids for gene silencing in mammalian cells. This opposing promoter system also provides a basis for generating randomised RNAi libraries in which random double-stranded DNA oligonucleotides can be introduced between the convergent U6 promoters. The expansion of this design to include two different RNA

polymerase III promoters in opposing orientations, with random oligonucleotide sequences between the convergent promoters, would produce a randomised RNAi library expressing functional siRNAs in mammalian cells and containing no inverted repeat sequences. Such genome-wide RNAi libraries would be useful for performing forward genetic screens similar to those reported using randomised ribozyme libraries (Kawasaki, H., Onuki, R., Suyama, E. and Taira, K. (2002) *Nature Biotech* 20:376-380) and universal peptide libraries (Xu, X., Leo, C., Jang, Y., Chan, E., Padilla, D., Huang, B.C.B., Lin, T., Gururaja, T., Hitoshi, Y., Lorens, J.B., Anderson, D.C., Sikic, B., Luo, Y., Payan, D.G. and Nolan, G.P. (2001) *Nature Genetics* 21:23-29). A significant advantage in using randomised RNAi libraries, over other nucleic acid-based libraries, in forward genetic approaches in mammalian cells would be the identification of 21 bases of complete sequence complementarity to the intracellular target RNA that is linked to the modified cellular phenotype. This length of sequence conservation could be used to more effectively identify candidate genes using homology-based search tools. In addition, these sequences could be chemically synthesised and used as tools for further validation of the identified targets or as potential therapeutics.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Johnson & Johnson Research Pty Ltd
Patent Attorneys for the Applicant:

BLAKE DAWSON WALDRON
PATENT SERVICES

REFERENCES

Asubel F M et al (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York N.Y.

Baker, M. W., and Macagno, E. R. (2000) *Curr Biol* 10(17), 1071-4.

Baulcombe, D. C. (1996) *Plant Mol Biol* 32(1-2), 79-88.

Beaucage and Caruthers (1981) *Tetrahedron Letts.* 22(20):1859-1862

Berkow et al eds., (1987) The Merck Manual of Diagnosis and Therapy, 15th Ed., , Rahway, N.J., pp 1206-1228.

Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) *Proc. Natl Acad Sci* 98, 14428-14483.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002) *Science* 296, 550-553.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., and Morgan, R.A. (2001) *Proc. Natl. Acad. Sci.* 98, 9742-9747.

Cogoni, C., and Macino, G. (1999) *Nature* 399 (6732), 166-9.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001) *Genes & Dev* 15:188-200.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) *Nature* 411(6836), 494-8.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) *Nature* 391(6669), 806-11.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000) *Nature* 404: 293-96

Hammond, S.M., Caudy, A.A., and Hannon, G.J. (2001) *Nat. Rev. Genet.* 2, 110-19.

Hsu, J.Y., Reimann, J. D. R., Sorensen, C.S., Lucas, J., and Jackson, P. K. (2002) *Nature Cell Biol.* 4, 358-366.

Hutvagner et al, A cellular function for the RNA-interference enzyme dicer in the maturation of the let-7 small temporal RNA. (2001) *Science* 293: 834-838.

Kawasaki, H., Onuki, R., Suyama, E. and Taira, K. (2002) *Nature Biotech* 20:376-380.

Kennerdell, J. R., and Carthew, R. W. (1998) *Cell* 95(7), 1017-26.

Kumar, M., and Carmichael, G. G. (1998) *Microbiol Mol Biol Rev* 62(4), 1415-34.

Lohmann, J. U., Endl, I., and Bosch, T. C. (1999) *Dev Biol* 214(1), 211-4.

Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, Methods in Enzymology 65:499-560.

Needham-VanDevanter et al (1984) *Nucleic Acids Res.*, 12:6159-6168.

Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998) *Proc Natl Acad Sci U S A* 95(25), 14687-92.

Paddison, P., Caudy, A. A., and Hannon, G.J. (2002) *Proc. Natl Acad. Sci.* 99, 1443-1448.

Paul, C.P., Good, P.D., Winer, I, and Engelke, D.R. (2002) *Nature Biotech* 20, 505-508.

Pearson and Regnier (1983) *J. Chrom.* 255:137-149.

Raponi, M., Dawes, I.W., and Arndt, G.M. Characterization of flanking sequences using long inverse PCR. (2000) *Biotechniques* 28, 840-844.

Sambrook et al (1989) Molecular Cloning, A laboratory Manual, Cold Spring Harbor Press, Plainview N.Y.

Schoppmeier, M., and Damen, W. G. (2001) *Dev Genes Evol* 211(2), 76-82.

Svoboda, P., Stein, P., Hayashi, H., and Schultz, R. M. (2000) *Development* 127(19), 4147-4156.

Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A., and Driscoll, M. (2000) *Nat Genet* 24(2), 180-3.

Thompson, B., Tonwsley, F., Rosin-Arbesfeld, R., Muisi, H., and Bienz, M. (2002) *Nature Cell Biol.* 4, 367-373.

Wargelius, A., Ellingsen, S., and Fjose, A. (1999) *Biochem Biophys Res Commun* 263(1), 156-61.

Wianny, F., and Zernicka-Goetz, M. (2000) *Nat Cell Biol* 2(2), 70-5.

Yang, S., Tutton, S., Pierce, E., and Yoon, K. (2001) *Mol Cell Biol* 21(22), 7807-16.

Xu, X., Leo, C., Jang, Y., Chan, E., Padilla, D., Huang, B.C.B., Lin, T., Gururaja, T., Hitoshi, Y., Lorens, J.B., Anderson, D.C., Sikic, B., Luo, Y., Payan, D.G. and Nolan, G.P. (2001) *Nature Genetics* 21:23-29.

FIGURE 1

FIGURE 2

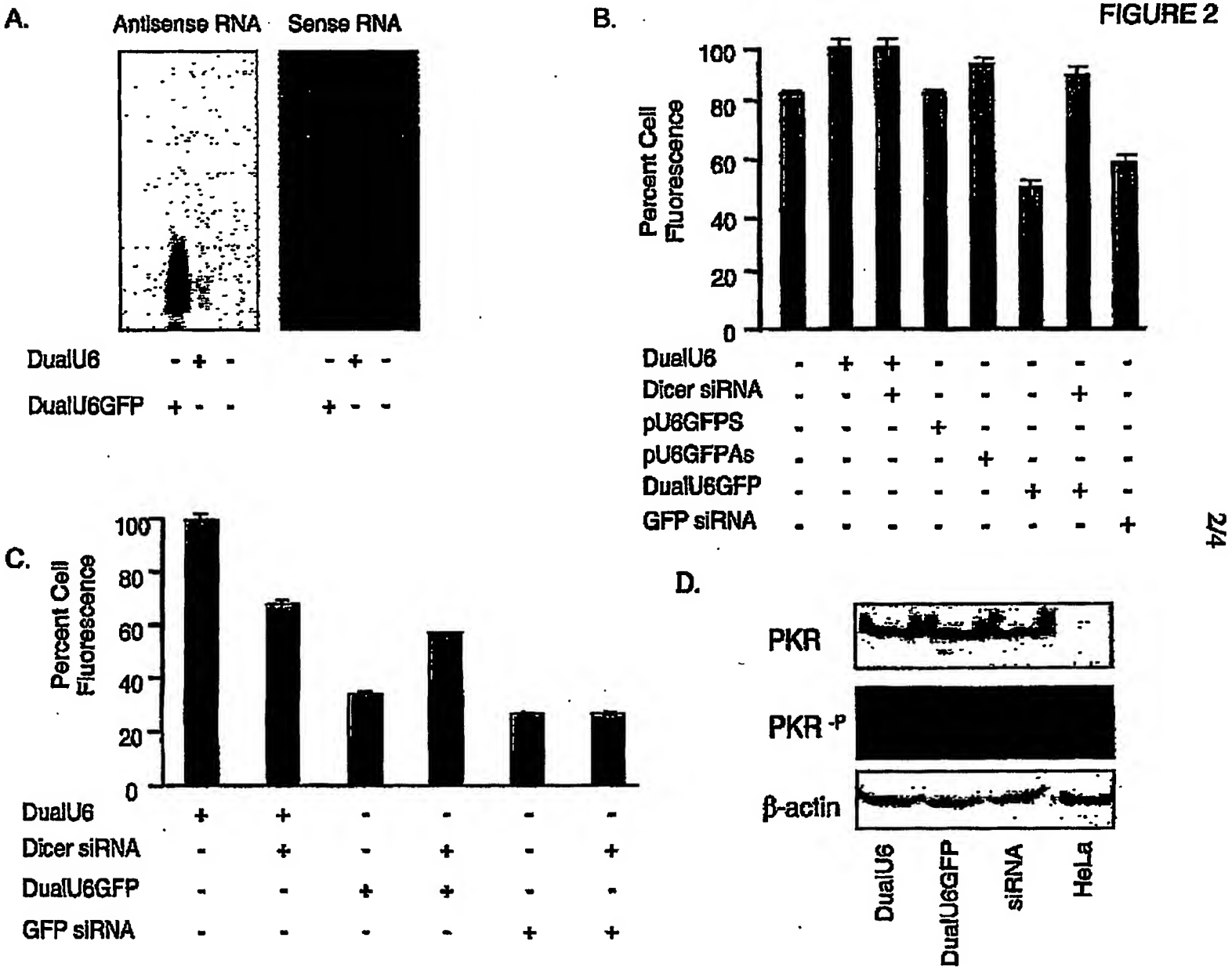

FIGURE 3

Figure 4. Suppression of dEGFP transgene expression using a stably integrated convergent transcription vector.